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Award Number: DAMD17-98-1-8303

TITLE: Alteration in the Nuclear Structure of Breast Cancer Cells in Response to ECM Signaling

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REPORT DATE: September 1999

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

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DTIC QUALITY INSPECTED 4

20010124 080

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1999	Annual Summary (17 Aug 98 -16 Aug 99)

4. TITLE AND SUBTITLE
Alteration in the Nuclear Structure of Breast Cancer Cells in Response to ECM Signaling

5. FUNDING NUMBERS
DAMD17-98-1-8303

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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release;
Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Eukaryotic chromosomes are thought to be separated into topologically independent loop domains by periodic attachment onto an intranuclear frame known as the nuclear matrix. Specific DNA sequences that bind to the nuclear matrix are called matrix attachment regions (MARs), in which a specialized DNA context sequences exhibiting high base unpairing propensity (BUR) is typically found. Besides organization of eukaryotic DNA, BURs/MARs may also be important for various functions including replication, transcription and recombination. A strong BUR-binding protein, p114, previously isolated from breast carcinoma cells, was identified as to be poly ADP-ribose polymerase (PARP). Depleting PARP by antisense construct in MDA-MB-231 cells resulted in the lost of aggressive phenotype *in vitro*, suggesting a critical role for PARP in the onset and/or maintenance of the malignant phenotype. Additionally, HMG I(Y) was also found to be BUR-binding protein, and its expression was well correlated with aggressive breast cancer cells. Using three directly comparable cell systems, we demonstrated that the level of HMG I(Y) expression is dynamically regulated in human breast cancer cells in response to varying types of signaling that affect metastatic ability, including the HRG-*erb*B pathway and those from extracellular matrix (ECM). Finally, to investigate changes in loop domain structure upon three-dimensional alveolar formation induced by ECM, β-casein gene was chosen here for future study.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
48

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

We identified a protein with 114 kDa (p114) from breast carcinoma cells that exhibit a strong binding activity to a specialized DNA context sequences that exhibit high base unpairing propensity. Such a base unpairing region (BUR) is typically found in nuclear matrix attachment regions (MARs); and are thus a hallmark thereof (reviewed in Kohwi-Shigematsu and Kohwi, 1997). BURs/MARs are thought to be important for functional organization of eukaryotic DNA (Gasser and Laemmli, 1987). The BUR-binding activity of this protein (p114) was detected in human breast tumor specimens, but it was virtually undetectable in normal breast counterparts (Yanagisawa et al., 1996). The activity correlated with progression of breast cancer: stronger activity was detected in more aggressive breast cancer than in more differentiated breast cancer. P114 was identified to be poly ADP-ribose polymerase (PARP) (Galand and Kohwi-Shigematsu, 1999). This finding that PARP can recognize primary sequences such as BURs was totally unprecedented (Galand and Kohwi-Shigematsu, 1999). We hypotheized that the interaction between PARP and MARs in breast cancer cells may be critical for the onset and/or maintenance of the malignant phenotype. My recent study showed that a very aggressive human breast cancer cell line, MDA-MB-231 when stably transfected with an antisense PARP expression construct, lost anchorage independent growth capability and its strong invasive phenotype *in vitro* (see preliminary results).

Additionally, a doublet of 20 kDa proteins were purified by a BUR affinity column from human breast carcinoma cells. These proteins were identified as the high mobility group protein, HMG I, and its splicing variant, HMG Y, which specifically bind BURs. We systematically analyzed whether a substantial increase in HMG I(Y) expression is indeed correlated with breast cancer cells that have metastasizing ability. We found this to be the case using three directly comparable systems: estrogen-dependent MCF-7 cells with and without heregulin cDNA expression; metastasizing MDA-MB-231 cells with and without antisense heregulin cDNA; and MDA-MB-231 cells before and after treatment with metalloproteinase B (MMP-9) chemical inhibitor. (please see attached manuscript entitled "HMG I(Y) Recognizes Base-Unpairing Regions of Matrix Attachment Sequences and Its Increased Expression is Directly Linked to Metastatic Breast Cancer Phenotype", Liu et al., in press)

We will study changes in loop domain structure upon three-dimensional alveolar formation induced by an extracellular matrix (ECM). For this purpose, we have selected the β-casein gene which expression requires the formation of an ECM-induced three-dimensional alveolar structure (reviewed in Roskelley et al., 1995). Using the genomic sequence information that corresponds the first exon of the β-casein gene, three BAC clones were obtained for the gene locus from the mouse BAC library (Genome System Inc.). Five potential BURs are identified from these BAC clones and under analysis.

In summary, the expression of BUR-binding proteins is well correlated with the aggressive phenotype of breast carcinoma cells. Specific binding to BURs in cancers may participate in gene regulation to trigger or maintain the malignant phenotype in response to signals from ECM, or during progression of tumorigenesis and differentiation. The research plan will provide an important insight into the fundamental mechanism underlying breast cancer development and/or maintenance, and may provide a clue for breast therapy in the future.

BODY OF REPORT

SPECIFIC AIM 1. To fully establish the correlation between p114 MAR-binding activity and the alteration of cell shape associated with either normal or malignant phenotype. To determine whether p114 MAR-binding activity is critical for the cause/or maintenance of the malignant phenotype.

PROGRESS 1: This p114 protein was identified to be poly ADP-ribose polymerase (PARP) which, besides DNA ends and nicks, can also recognize primary sequence of BUR (Galand and Kohwi-Shigematsu, 1999). My antisense approach showed that depleting PARP by expressing antisense PARP RNA in one of the most aggressive human breast cancer cells, MDA-MB-231, led to the loss of anchorage independent growth in soft agar, and a dramatic reduction in the invasive phenotype of these cells *in vitro* (my data are shown in the Preliminary Results). The immediate future experiment will be testing these antisense PARP cells in nude mice.

PRELIMINARY RESULTS:

1). A high level of PARP expression in breast carcinoma cells in not simply because these cells have a high rate of proliferation. MDA-MB-231/antisense-PARP cells showed similar proliferation as the wild type MDA-MB-231 cells in plastic culture dishes (Fig. 1A, 1B).

2). MDA-MB-231/antisense-PARP clones lost ability to grow in soft agar in an anchorage-independent fashion (Fig. 2A, 2B).

3). MDA-MB-231/antisense-PARP clones exhibit significantly reduced invasive activity *in vitro* by Boyden Chamber Assay (Fig. 3); and changed cell morphology when cells were grown in matrigel culture (Fig. 4).

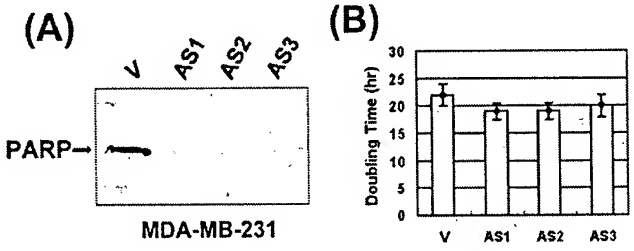

Figure 1. Western analysis of PARP and determination of doubling time for MDA-MB-231/V, AS1, AS2, and AS3 cells. (**A**) Twenty μg proteins extracted from V, AS1, AS2, and AS3 cells were used in the Western blot analysis using anti-PARP antibody. (**B**) Cell growths for V, AS1, AS2, and AS3 cells in culture were monitored over 5 days.

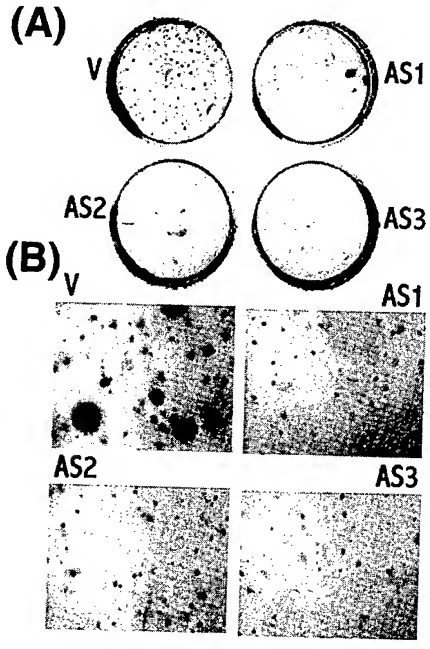

Figure 2. Decrease of anchorage independence growth by PARP antisense expression in MDA-MB-231 cells, V, AS1, AS2, and AS3. Soft-agar assays were performed with single wells of six-well dished by suspending 10,000 cells in 2 ml of 0.3% Noble agar in IMEM/5% FBS containing 400 μg/ml G418 and overlaying the suspension on 2.5 ml of presolidified 0.5% agar in the same medium. The cultures were then overlaid with 2 ml of 0.3% agar in the same medium, covered with another 2 ml medium and changed every 3 or 4 days. At the end of 2 weeks, colonies were stained with the vital stain 2-(4-iodophenyl)-5-phenyltetrazolium chloride (INT) (Sigma) for 72 h at 37°C (Kang and Krauss, 1996). (**A**) Actual cell dishes for vector control, V, and antisense colonies, AS1, AS2, and AS3. (**B**) Cells were photographed under a 200X magnification.

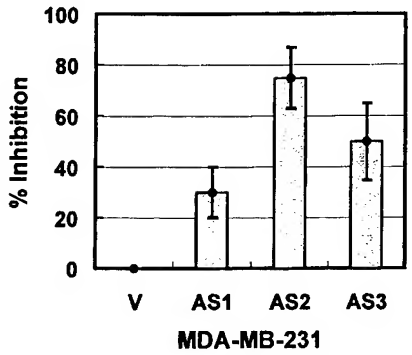

Figure 4. Reduction of the invasive phenotype of MDA-MB-231 upon PARP depletion by antisense. MDA-MB-231 series cells, V, AS1, AS2, and AS3, were tested in the Boyden chamber assay. Chemoinvasion was measured according to the number of cells transversing matrigel-coated filters. Data points present the mean from quadruplicate from a representative experiment. Standard deviation was calculated for each data point. This assay tends to give variable data due to difficulty in uniformly coating matrigel.

[**Boyden Chamber Assay:** Methodology for the Boyden chamber assay has been described extensively in previous publications (Thompson *et al.,* 1992). A chemoinvasion analysis was conducted with MDA-MB –231 cells in the Boyden chamber assay. Cells were plated at 10,000 cells/well (in quadruplicate) onto polycarbonate filters (12 μM pore, PVP free, Nucleopore, Pleaston, CA) coated with Matrigel (Collaborative Biochemical Products, Bedford, MA, 1:20 dilution of stock) for the chemoinvasion assays. NIH3T3 fibroblast conditioned media was used as a chemoattractant in the lower chambers and was prepared according to previously established protocols (Thompson *et al.,* 1992). Cells were incubated at 37^{0}C, 5% CO_2/95% air for 6h, filters were then fixed, stained with hematoxylin and cells on the top surface were removed. Filters were mounted onto glass slides and the number of cells which had migrated through the pores were assessed for each treatment group by image analysis systems or by microscopy.]

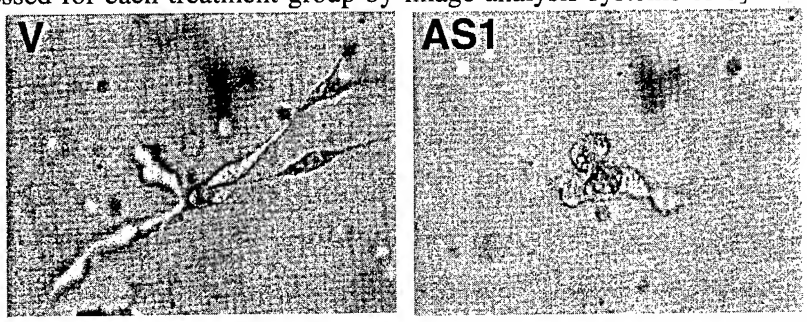

Figure 5. MDA-MB-231 (PARP antisense) cells have a spheroid-like cell morphology as opposed to stellar-like shape in control cells. [**Matrigel Outgrowth Assay:** MDA-MB-231 cells were plated at 50,000 cells mixed with 0.2 ml Matrigel (Becton Dickinson Inc.)/well on a present Matrigel layer (0.1 ml) in 12-well plate, covered by IMEM/5%FBS and grown for several days at 37^{0}C, 5% CO_2/95% air.]

Summary: *My preliminary results shown here strongly suggest that PARP may be a key player in the maintenance of the aggressive phenotype of breast maligancy.*

PROGRESS 2: During this study, I noticed that in the Southwestern blot, there is a small BUR-binding protein (around 20 kDa) was highly expressed in maligant breast cancer cells but not in nonmaligant cells. These doublet of 20 kDa proteins were purified by a BUR affinity column from human breast carcinoma cells and identified as the high mobility group protein, HMG I, and its splicing variant, HMG Y. We show that HMG I(Y) specifically binds BURs. Mutating BURs so as to abrogate their unwinding property greatly reduced their binding affinity to HMG I(Y). Numerous studies have indicated that elevated HMG I(Y) gene expression is correlated with more advanced cancers and with increased metastatic potential. We studied whether the expression of HMG I(Y) responds to signaling through the heregulin (HRG)-*erb*B pathway and the ECM. HMG I(Y) expression was increased in MCF-7 cells after stable transfection with an HRG expression construct, which led cells to acquire estrogen independence and metastasizing ability. A high level of HMG I(Y) expression was detected in metastatic MDA-MB-231 cells, but the expression was virtually diminished and the metastasizing ability was lost after cells were stably transfected with an antisense HRG cDNA construct. HMG I(Y) was also decreased in MDA-MB-231 cells when treated with a chemical inhibitor for MMP-9 that led to a reduction of invasive

capability *in vitro*. The level of HMG I(Y) expression, therefore, is dynamically regulated in human breast cancer cells in response to varying types of signaling that affect metastatic ability, including the HRG-*erb*B pathway and those from ECM (attached manuscript; Liu et al., in press).

CHANGE: P114 was identified as PARP which is known as an enzyme that binds to DNA ends and nicks generated upon DNA damage and contributes to the maintenance of genome stability (reviewed in Jeggo, 1998). It will be difficult to identify the BUR sequences bound to PARP *in vivo*. Therefore, we have selected the β-casein gene for studying changes in loop domain structure upon three-dimensional formation in response to ECM. The expression of β-casein requires the formation of an ECM-induced three-dimensional alveolar structure (reviewed in Roskelley et al., 1995), which provides an example for up-regulation in response to ECM. For this study, I will use a homogenous murine mammary epithelial cell line, SCp2 (Desprez et al., 1993), which was cloned from a heterogenous culture derived from mid pregnancy mammary glands. SCp2 cells grow well on tissue culture plastic in serum-containing medium. However, when given an exogenous basement membrane and lactogenic hormones in serum-free medium, they cease proliferation form alveolus-like structures, and functionally differentiate, as judged by the expression and secretion of milk proteins. Therefore, with this system, I will be able to examine how higher order chromatin structure changes in response to ECM-induced differentiation that accompanies a distinct morphology of breast epithelial cells.

Using the genomic sequence information that corresponds the first exon of the β-casein gene, three BAC clones were obtained for the gene locus from the mouse BAC library (Genome System Inc.). Using SATB1 and anyi-SATB1 antibody, five potential BURs already were isolated from these BAC clones and under analysis (data not shown). Even though SATB1 is a thymocyte factor, it can bind to the BUR in any cell system *in vitro* and therefore can be used to clone BUR. Precise methods for this experiment are described in our recent publication (Kohwi-Shigematsu et al., 1997). Once a BUR is identified, I will determine by *in situ* hybridization whether this site is anchored or not on the nuclear matrix of SCp2 cells when cultured on ECM to form alveolar structure. This will be compared with *in situ* hybridization with the SCp2 nuclear matrix when the cells were cultured on plastic (without alveolar structure). The result will tell me whether anchoring of this particular BUR is a specific event in response to ECM induced three-dimensional structure. Because I already know the expression status of these genes in response to ECM, I can immediately make a correlation with the loop domain structure and gene expression. As a future experiment, provided that positive results were obtained from the previous experiments, I will explore specific proteins that bind to the BURs isolated. To do this, I will prepare a DNA affinity column using a particular BUR. Protein extracts from ECM-induced SCp2 cells will be used to purify such a protein. This strategy has been published by our laboratory (Kohwi-Shigematsu et al., 1997)

SPECIFIC AIM 2. A set of genomic sequences bound to p114 in vivo in T4-2 cells grown in EHS matrix will be obtained. The association of these sequences with nuclear matrix prepared from T4-2 cells either grown in EHS matrix (tumor phenotype) or where treated with β1-integrin antibody (non-malignant phenotype) will be compared.
[N/A in this period]

SPECIFIC AIM 3. For selected genomic sequences bound to p114 in T4-2 cells grown in EHS culture, specific genes will be identified nearby such genomic sequences. We will determine whether these specific genes expression is regulated by p114 MAR-binding activity altered by ECM signalling.
[N/A in this period]

LITERATURE CITED

de Belle, I., Cai, S., and Kohwi-Shigematsu, T. The genomic sequences bound to special AT-rich sequence-binding protein 1 (SATB1) in vivo in Jurkat T cells are tightly associated with the nuclear matrix at the bases of the chromatin loops. J. Cell Biol., *141:* 335-348, 1998.

Desprez, P. Y., Roskelley, C. D., Campisi, J., and Bissell, M. J. Isolation of functional cell lines from a mouse mammary epithelial cell train; the important of basement membrane and cell-cell interaction. Mol. Cell. Differ. *1:* 99-110, 1993.

Galand, S. A., and Kohwi-Shigematsu, T. Poly (ADP-ribose) polymerase and Ku autoantigen form a complex and synergistically bind to matrix attachment sequences. J. Biol. Chem. *274:* 20521-20528,1999.

Gasser, S. M., and Laemmli, U. K. A glimpse at chromosomal order. Trends Genet., *3:* 16-22, 1987.

Jeggo, P. A. DNA repair: PARP - another guardian angel?. Curr. Biol., *8:* R49-R51, 1998.

Kang, J. S., and Krauss, R. S. Ras induces anchorage-independent growth by subverting multiple adhesion-regulated cell cycle events. Mol. Cell. Biol., *16:* 3370-80, 1996.

Kohwi-Shigematsu, T., and Kohwi, Y. *In:* R. Curtis Bird (ed.) Nuclear Structure and Gene Expression: High unwinding capability of matrix attachment regions and ATC-sequence context-specific MAR-binding proteins, pp. 111-144. San Diego: Academic Press, 1997.

Liu, W.-M., Guerra-Vladusic, F. K., Kurakata, S., Lupu, R., and Kohwi-Shigematsu, T. HMG I(Y) recognizes Base-Unpairing regions (BURs) of matrix attachment regions and is directly linked to metastatic breast cancer phenotype. Cancer Research (in press).

Roskelley, C. D., Srebrow, A., and Bissell, M. J. A hierarchy of ECM-mediated signaling regulates tissue-specific gene expression. Curr. Opin. Cell Biol., *7:* 736-47, 1995.

Thompson, E. W., Paik, S., Brünner, N., Sommers, C. L., Zugmaier, G., Clarke, R., Shima, T. B., Torri, J., Donahue, S., and Lippman, M. E. Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. J. Cell. Phys., *150:* 534-44, 1992.

Yanagisawa, J., Ando, J., Nakayama, J., Kohwi, Y., and Kohwi-Shigematsu, T. A matrix attachment region (MAR)-binding activity due to a p114 kilodalton protein is found only in human breast

APPENDICES

1. Research accomplishments:
   a. One publication in Cancer Research (in press) "HMG I(Y) Recognizes Base-Unpairing Regions of Matrix Attachment Sequences and Its Increased Expression is Directly Linked to Metastatic Breast Cancer Phenotype".

   b. Important preliminary results supporting my hypothesis that PARP (p114) may be a key player in the maintenance of the aggressive phenotype of breast maligancy.

   c. Potential BURs isolated from β-casein gene locus.

2. Attached manuscript "HMG I(Y) Recognizes Base-Unpairing Regions of Matrix Attachment Sequences and Its Increased Expression is Directly Linked to Metastatic Breast Cancer Phenotype"

HMG I(Y) Recognizes Base-Unpairing Regions of Matrix Attachment Sequences and Its Increased Expression is Directly Linked to Metastatic Breast Cancer Phenotype.

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Running title: A link of MAR-binding proteins to breast cancer metastasis.

Key words: HMG I(Y), matrix attachment region, heregulin, breast cancer and metastasis.

Abstract

Base unpairing regions (BURs) are specialized DNA contexts that confer an exceptionally high unwinding propensity, and are typically identified within various matrix attachment regions (MARs). A BUR affinity column was used to purify a doublet of 20 kDa proteins from human breast carcinoma cells. These proteins were identified as the high mobility group protein, HMG I, and its splicing variant, HMG Y. We show that HMG I(Y) specifically binds BURs. Mutating BURs so as to abrogate their unwinding property greatly reduced their binding affinity to HMG I(Y). Numerous studies have indicated that elevated HMG I(Y) gene expression is correlated with more advanced cancers and with increased metastatic potential. We studied whether the expression of HMG I(Y) responds to signaling through the heregulin (HRG)-*erb*B pathway and the extracellular matrix (ECM). HMG I(Y) expression was increased in MCF-7 cells after stable transfection with an HRG expression construct, which led cells to acquire estrogen independence and metastasizing ability. A high level of HMG I(Y) expression was detected in metastatic MDA-MB-231 cells, but the expression was virtually diminished and the metastasizing ability was lost after cells were stably transfected with an antisense HRG cDNA construct. HMG I(Y) was also decreased in MDA-MB-231 cells when treated with a chemical inhibitor for MMP-9 that led to a reduction of invasive capability *in vitro*. The level of HMG I(Y) expression, therefore, is dynamically regulated in human breast cancer cells in response to varying types of signaling that affect metastatic ability, including the HRG-*erb*B pathway and those from ECM.

Introduction

Specialized genomic DNA sequences that have a high affinity to the isolated nuclear matrix *in vitro* have been designated as matrix/scaffold attachment regions (MARs/SARs) (1-6). MARs were thought to tether chromatin onto the nuclear matrix, thus separating genomic DNA into topologically independent loop domains which may be important for various functions including transcription and replication. For instance, MARs have been shown to be directly linked to biological activity such as cell-type specific regulation (7), demethylation (8) and chromatin accessibility (9).

MARs typically contain a region with a specialized context that possesses an exceptionally high propensity to unwind by contiguous base-unpairing when subjected to negative supercoiling (10, 11). Such a region is referred to as a BUR (base-unpairing region) and typically contains clusters of ATC sequence stretches, a specific sequence context in which one strand exclusively consists of mixed A, T, and C nucleotides (ATC sequences) (reviewed in 12). BURs, when they are in a double-stranded form, are specific targets of cell type-specific factors such as SATB1 predominantly expressed in thymocytes (13) and a B cell factor, Bright (14). Recently, genomic DNA sequences that bind *in vivo* to SATB1 were isolated, and these sequences were shown to be localized at the bases of chromatin loops *in vivo* (15). BURs are also preferential targets of other proteins including nucleolin (16), mutant p53 (17), and p114 which is found in breast carcinoma cells (18). When a BUR is mutated to abrogate the unwinding capability, its affinity to SATB1 is abolished (13) and its affinity to the nuclear matrix is greatly reduced (11).

A group of proteins may exist that bind specifically to BURs in cancer cells and participate in gene regulation to trigger or maintain the malignant phenotype. Here we searched for a BUR-binding protein, the expression of which is well correlated with the aggressive phenotype of breast carcinoma cells that have the metastasizing ability. We detected two proteins that migrate as a doublet on SDS-PAGE at 20 kDa molecular weight, the BUR-binding activity of which is dramatically elevated in highly metastatic breast carcinoma cell lines, but is much less in non-

metastatic breast carcinoma cell lines. The 20 kDa proteins were identified as HMG I(Y), a member of the high-mobility group (HMG) non-histone chromatin proteins. HMG I and HMG Y are splice variants of the same gene located on human chromosome 6p21 (19), which have been demonstrated to strongly bind SARs/MARs (20). Here, we found that HMG I(Y), the combination of HMG I and the splice variant HMG Y, exhibits a strict preference for double-stranded BUR sequences, the key structural element of MARs, over its mutated version that is still AT-rich but has lost the unwinding propensity.

In general, most normal differentiated mammalian cells and adult tissues express extremely low levels of HMG I(Y) mRNA and protein (reviewed in 21). Overexpression of HMG I(Y) was first shown in HeLa cells (22), and since then in various types of tumors in mice and rats (23-28). Interestingly, an antisense construct for HMG I-C, another AT-hook containing HMG protein, has been shown to prevent neoplastic transformation of rat thyroid cells by retroviral oncogenes, and this led to the elimination of the endogenous level of HMG I(Y) proteins (29). Human thyroid neoplasias and thyroid carcinoma cell lines express HMG I(Y) at a high level, while benign follicular adenomas, goiters, and normal thyroid cells do not (30). HMG I(Y) expression has also been observed to increase in various types of human cancer, including prostate cancer (31, 32), colorectal carcinomas (33), uterine leiomyomata (34) and squamous intraepithelial and invasive lesions of the uterine cervix (35). Apparently, HMG I(Y) gene expression associates with the malignant phenotype of human cells and tissues.

Numerous studies have indicated that growth factors and their receptors play an important role in cancer biology. The EGF-like receptor (EGFR) family is a group of transmembrane tyrosine kinases, that is frequently overexpressed in a variety of carcinomas (36-39), and high EGFR levels are associated with poor clinical prognosis (reviewed in 40). Holth et al. (41) have observed an EGF-induced over-expression of HMG I(Y) in the highly metastatic Hs578T, but not in the nonmetastatic MCF7 cells. It suggested that HMG I(Y) may have an important implication concerning the cellular mechanisms involved in the progression of mammary epithelial tumors. Besides EGFR, amplification and overexpression of one of the *erb*B-receptors, *erb*B-2, also

correlates with a poor prognosis in some adenocarcinomas, most notably breast cancer (36, 42-48). The activator for *erb*B-2, heregulin (HRG) originally called gp30 (49), has been suggested to play an important role in breast tumor development (50). HRG is a growth factor originally isolated from hormone independent and invasive breast cancer cells (51, 52). MCF-7 cells can be converted to a more aggressive phenotype and rendered tumorigenic and metastatic *in vivo* merely by transfecting them with a HRG expression construct (53). On the other hand, transfecting highly metastatic MDA-MB-231 cells with an antisense-HRG construct led to an inhibition of tumorigenic phenotype (54). In order to further address the role of HMG I(Y) in carcinogenesis and to examine the effect of HRG on HMG I(Y) expression, we have undertaken studies in these two cell models of human breast cancer progression. In both cases, the higher level of HMG I(Y) protein was found to be strictly correlated with the high expression of HRG and metastasizing capability of cells.

Proteases that degrade the extracellular matrix ECM, including the serine proteases and matrix metalloproteases (MMP) gelatinase A (MMP-2) and B(MMP-9), have been implicated in various pathological states of growth, invasion and metastasis (55, 56). Inhibition of MMP-9 expression using a ribozyme has been shown to block metastasis in the rat sarcoma model system (57). We report here that blocking MMP-9 activity in MDA-MB-231 cells with an MMP-9 chemical inhibitor resulted in a loss of invasive phenotype *in vitro*, and a decrease of the HMG I(Y) protein levels. Our observation suggests that the expression of HMG I(Y) can be regulated dynamically in response to various types of signaling that affect metastastic ability, including heregulin and ECM.

Materials and Methods

Cell Lines and Cell Extract Preparation. Breast carcinoma cell lines MDA-MB-453, SK-BR-3, BT-474, MCF-7, Hs578T, BT-549, and MDA-MB-231, were obtained from American Type Culture Collection (Rockvile, MD). These cell lines were maintained in IMEM containing 10% fetal bovine serum (Gibco BRL). MCF-7/HRG cells were maintained as described (53). IMEM Phenol Red free media and charcoal-stripped serum were used for experiments looking at the effect of 17β-estradiol in MCF-7 cells. Cells were stimulated for 48 h with Estradiol (E_2, 10^{-9}M) (53), Tamoxifen (Tam, 10^{-7}M) (53) or anti-estrogen ICI 164,384 (ICI, 10^{-7}M) (58). MDA-MB-231/AS cell lines were maintained as MDA-MB-231 cells except for the addition of 200 μg/ml G418 (Geneticin, Sigma) into medium. Metalloprotease MMP-9 inhibitor (N-methyl-(3S)-S-[(2R)-2-hydroxyaminocarbonylmethyl-I-oxoundecyl]-hexahydropyridazine-3-carboxamide, a gift from Sankyo Pharmaceutical Company, Tokyo, Japan) was added to MDA-MB-231 cell cultures at the indicated concentrations (2.5, 5 and 10 μM) for six days. *In situ* cell death detection kit (Boehringer Mannheim) was used for TUNEL assay. Cell extracts were prepared as described (16). Briefly, the cell pellet was resuspended in approximately 5X volume of Dignam's extraction buffer [0.42 M NaCl, 20 mM Hepes (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1mM DTT, 0.1 mM Phenylmethylsulfony fluoride, and 10 μg/ml each of leupeptin and aprotinin]. Cells were lysed in a Dounce homogenizer (50 strokes with pestle A) and followed by a brief sonication. The extract was centrifuged for 20 min in a TLA-100 rotor in a Beckman TL-100 ultracentrifuge at 100,000 rpm, and the supernatant was either assayed directly using Southwestern and Western analysis or used for affinity purification of MAR-binding proteins.

Boyden Chamber Assay. Methodology for the Boyden chamber assay has been described extensively in previous publication (58). A chemoinvasion analysis was conducted with MDA-MB –231 cells in the Boyden chamber assay. Cells were plated at 10,000 cells/well (in

quadruplicate) onto polycarbonate filters (12 μM pore, PVP free, Nucleopore, Pleaston, CA) coated with Matrigel (Collaborative Biochemical Products, Bedford, MA, 1:20 dilution of stock) for the chemoinvasion assays. MMP-9 inhibitor (0, 0.1, 0.5, 1, 5, 10, 15, 20 μM) was added to the upper chamber and lower chambers. NIH3T3 fibroblast conditioned media was used as a chemoattractant in the lower chambers and was prepared according to previously established protocols (58). Cells were incubated at 37^{0}C, 5% CO_2/95% air for 6 h, filters were then fixed, stained with hematoxylin and cells on the top surface were removed. Filters were mounted onto glass slides and the number of cells which had migrated through the pores were assessed for each treatment group by image analysis systems or by microscopy.

DNA Affinity Purification of MAR-binding Proteins and Identification. The MAR affinity column was prepared exactly as described (Dickinson and Kohwi-Shigematsu, 1995). Briefly, the wild-type complementary oligonucleotides, 5'TCTTTAATTTCTAATATA-TTTAGAAttc3' and 5'TTCTAAATATATTAGAAATTAAAGAgaa3', were annealed, phosphorylated at 5' ends, and ligated to form concatemers (lowercase type indicates single-strand overhangs). Routinely, 200 μg of the double-stranded oligomers was coupled to 1 ml of cyanogen bromide-activated Sepharose 6 MB (Pharmacia). The oligonucleotide 5'TCTTTAATTTCTACTGCTTTAGAAttc3' and its complementary strand 5'TTCT-AAAGCAGTAGAAATTAAAGAgaa3' were used for generating a mutated MAR DNA affinity column. Cell extract was prepared from approximately 10^9 cells, incubated with salmon sperm DNA competitors, centrifuged, and diluted with buffer Z [25 mM HEPES (pH7.9), 1.25 mM $MgCl_2$, 1mM DTT, 20% glycerol, and 0.1% (v/v) NP40] as described previously (18). Cell extract was re-adjusted to 0.1M KCl in buffer Z, loaded on a mutated MAR DNA affinity column, and washed with the same buffer. The bound proteins were eluted with buffer Z containing 0.2, 0.3, 0.4, 0.6 and 1.0 M KCl, and this MAR-binding protein was eluted at 0.3 M KCl. The eluted fraction was re-adjusted to 0.1 M KCl in buffer Z, loaded on a wild-type MAR DNA affinity column, and washed with buffer Z containing 0.1 and 0.2 M KCl. The bound proteins were eluted

at 0.3, 0.4, 0.5, 0.6, 0.8 and 1.0 M KCl, and this MAR-binding protein was eluted at 0.4-0.6 M KCl. The purified p20 was sent to the Protein Structure Laboratory at University of California at Davis for partial amino acid sequencing analysis.

RT-PCR and DNA Constructs. Total cellular RNA was extracted from MDA-MB-231 cells by a one-step acid guanidinum isothiocynate-phenol procedure using Tri Reagent (Sigma), precipitated with ethanol, and quantitated by spectrophotometry. Five μg total RNA was used for RT-PCR as instructed by the manufacturer (GIBCO BRL). Two hundred units of Super Script II, Rnase H⁻ Reverse Transcriptase, was added in a typical 20 μl reaction (50 ng specific primer, 5 μg total RNA, 1X First Strand Buffer, 10 mM DTT and 0.5 mM dNTP), and incubated 1 h at 42°C. The reaction was inactivated by heating at 70°C for 15 min. To remove RNA complementary to the cDNA, RNase H was added and incubated at 37°C for 30 min. The purified cDNA was used as a template for amplification in PCR. The primers including BamHI linker for amplification of HMG I(Y) cDNA are 5'-gaaggatccATGAGTGAGT-CGAGCTCGAAGT-3' and 5'-cagggatccAAGGAAGCTGCTCCTCCAGTGA-3'. PCR was then performed in a 50 μl volume including 2.5 units of Taq polymerase, 10x buffer (Promega), 1.5 mM $MgCl_2$, 200 μM dNTPs, and 2 μM of each primer under the following conditions: 94°C for 5 min; followed by 30 cycles of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 45 sec; followed by 5 min at 72°C. The 372 bp BamHI fragment of HMG I cDNA and the 339 bp BamHI fragment of HMG Y cDNA were cloned into the pGEX-4T-1 vector (Strategene) to express GST-HMG I(Y) proteins. GST-fusion proteins were synthesized in *Escherichia coli* and purified as described (59). Protein concentrations were determined by using a protein assay kit (Bio-Rad) and by comparing to BSA standards on SDS-PAGE gel stained with commassie blue.

Southwestern and Western Analysis. Southwestern and Western blots were done basically as described previously (16) with some modifications. For Southwestern analysis,

protein samples were adjusted to 1X SDS-PAGE loading buffer and incubated at 37^0C for 10 min before being applied to a 15% polyacrylamide mini gel layered with 3.75% stacking gel. After electrophoresis, separated proteins were transferred electrophoretically at 45 V/cm at 0.75 mA for 1 h onto Immobilon P membrane (Millipore). The membrane was blocked with 5% BSA in the binding buffer [20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM DTT, and 0.25% BSA] at RT for 1-2 h. To 20 ml of binding solution, heat-denatured salmon sperm DNA to a final concentration of 50 μg/ml was added, and the membrane was preincubated at RT for 30 min. ^{32}P-labeled probe (approximately 10^8 cpm/μg DNA) was added and further incubated for 40 min at RT. The membrane was washed four times for 10 min in binding buffer. It was then exposed to XAR film. For Western blot analysis, proteins separated by SDS-PAGE were electrophoretically transferred to Immobilon P membranes (Millipore) in 20 mM sodium phosphate buffer (pH 6.8). Prestained broad range protein marker (Bio-Rad) was included for internal molecular mass standards. The filter was blocked in 5% BSA in TST [20mM Tris-HCl (pH 7.6), 0.5 M NaCl, and 0.05% Tween20], washed in TST buffer minus BSA, and incubated at RT for 1.5 h with either polyclonal antibody to HMG I(Y) (from Santa Cruz Biochemicals or a generous gift obtained from Dr. Vincenzo Giancotti) at a 1: 200 dilution, or monoclonal anti-β-actin antibody (Sigma). After being washed in TST, the filter was incubated with a 1:10,000 dilution of rabbit anti-goat, goat anti-rabbit or mouse IgG horseradish peroxidase conjugate. Following extensive washing in TST, the blots were incubated with ECL (enhanced chemiluminscence) reagent solutions (PIERCE) and exposed to XAR film for visualization of protein bands. Quantitation of Southwestern and Western blots were done individually by phosphorimager and densitometer (Storm 860, Molecular Dynamics).

Gel-mobility Shift Assay. The experiment was performed basically as described (16). Binding reactions were done in 20 μl total volume containing 10 mM Hepes (pH 7.9), 1 mM DTT, 50 mM KCl, 2.5 mM $MgCl_2$, 10 % glycerol, 1 μg double-stranded poly (dI-dC), 10 μg BSA and

2.5 ng –120 ng GST-HMG I or Y protein. Samples were preincubated at room temperature (RT) for 5 min before radiolabeled DNA probe was added. The Bam HI-Hind III fragments of wild-type $(25)_7$ and mutated $(24)_8$ (13), or the EcoRI fragments of wild-type and mutated IgH 3'MAR (5), were end labeled by the klenow fragment of DNA polymerase and used as DNA probes in the present study.

Results

Increased MAR-binding Activity of Small Proteins (20 kDa) in Metastatic Breast Cancer Cells. To investigate BUR-binding proteins that might be correlated with the aggressive phenotype of breast carcinomas, we chose to examine seven human breast carcinoma cell lines (listed in Table 1). High levels of HRG expression have been observed in aggressive breast cancer cells (Hs578T, BT-549 and MDA-MB-231) that are vimentin positive/estrogen receptor (ER)-negative, invasive on matrigel outgrowth assay, and metastatic *in vivo* in nude mice (50, 60, 61). In contrast, MDA-MB-453, SK-BR-3, BT-474 and MCF-7 are representatives of non-metastatic breast cancer cell lines with no detectable level of heregulin (HRG) (50, 60, 61) or vimentin proteins (58). Using whole cell extracts from these cells, we performed Southwestern analysis using the synthetic MAR probe, wild type $(25)_7$. This multimer contains the 25 bp sequence that was derived from a MAR 3' of the IgH enhancer and contains the core unwinding element (10). Wild type $(25)_7$ has a base-unpairing property, thus it is a BUR, and binds to the nuclear matrix with high affinity (11). Interestingly, the BUR-binding activity of small proteins (Fig. 1, 20 kDa, arrowhead) is remarkably higher in aggressive metastatic cells (Fig. 1, lane 5 - lane 7) as compared to non-metastatic cells (Fig. 1, lane 1 - lane 4). Three bands were seen at small protein range, a doublet p20(s) and a very faint band around 15 kDa. Larger proteins on the same Southwestern blot exhibited an overall similar and relatively weaker BUR-binding activity (Fig. 1). This result suggests a possible correlation between increased BUR-binding activity of small proteins and the progression of breast cancer cells to a metastatic phenotype. The same Southwestern blot was directly subjected to Western blot analysis using anti-β-actin antibody (Fig. 1, bottom panel). Each sample was precisely quantitated for its protein amount before loading. There was some variation in the β-actin expression depending on different cell lines. However, the strong MAR-binding activity detected in metastatic cells, especially MDA-MB-231, is

apparently not due to an excess protein load (compare Fig. 1, lanes 1, 2 to lanes 5, 7, for example).

Southwestern blot analysis using a mutated $(24)_8$ probe was performed for MDA-MB-231 cells. This mutated DNA was derived from the original 25 bp sequence by specifically mutating the core unwinding element to abrogate the unpairing propensity (10). The 20 kDa proteins bound poorly to the mutated $(24)_8$ on the Southwestern blot (data not shown), suggesting that these proteins are BUR-binding proteins similar to SATB1 (13) and nucleolin (16).

Purification and Identification of a 20 kDa MAR-binding Protein from MDA-MB-231 Cells. To purify 20 kDa MAR-binding proteins, wild-type and mutated MAR DNA affinity columns were prepared by coupling multimers of either the wild-type 25 mer oligonucleotide or the mutated 24 mer oligonucleotide to activated Sepharose as described in Materials and Methods. The strategy for purifying this p20(s) from MDA-MB-231 cell extracts is first to allow p20(s) to bind the multimerized mutated (24)n DNA affinity column by passing through the column three times under low salt conditions. Then, the eluted fractions were applied onto the multimerized wild-type $(25)_n$ DNA affinity column. Following this protocol, a major band of 20 kDa was purified by BUR-affinity chromatography and stained with commassie blue (Fig. 2A). The purified protein and proteins in the crude extract were analyzed side-by-side by Southwestern blot analysis, confirming that the 20 kDa BUR-binding protein was purified (Fig. 2B). Previously identified p114 (18) were purified from the same column at higher salt concentrations beyond 0.6M. Sequencing of two peptide fragments obtained by tryptic digestion of the gel-purified 20 kDa protein, KQPPVSPGTALVGSOK and EPSEVPTPK, revealed their identity with the published sequence of human HMG I (62). HMG I (11 kDa) migrates as a 20 kDa protein in a 15% SDS-PAGE gel. Western blot analysis using anti-HMG I(Y) antibody indicated that increased MAR-binding activity detected in human metastatic breast cancer cells (Fig 1, lane 5 - lane 7) was due to the elevated HMG I protein (see Fig. 2C). An additional protein that migrates slightly faster than HMG I is also recognized by anti-HMG I(Y) after longer exposure (see Fig. 2C, bottom panel). This is most likely an isoform of HMG I, the HMG Y, which is produced by

alternative splicing of mRNA transcripts from a single gene. This may explain the appearance of a doublet on the Southwestern blot around *Mr.* 20,000 (Fig.1). We therefore conclude that the two MAR-binding proteins that migrate close to 20kDa from MDA-MB-231 cells are HMG I(Y).

GST-HMG I(Y) Fusion Proteins Selectively Bind BURs. To prepare glutathione-S-transferase (GST)-fused HMG I and HMG Y proteins, HMG I(Y) cDNAs were cloned by RT-PCR strategy using RNA from MDA-MB-231 cells. The 372 bp fragment of HMG I cDNA and the 339 bp fragment of HMG Y cDNA were separately cloned into the pGEX-4T-1 vector (Stratagene). GST-HMG I(Y) fusion proteins purified from bacteria were examined by gel mobility shift assay using wild-type $(25)_7$ and mutated $(24)_8$ (Fig.3A). Both GST-HMG I and HMG Y bound with strong affinity to wild-type $(25)_7$, with an estimated dissociation constant (kd) in the range of 4×10^{-9} M (Fig. 3A, upper panel) under conditions of protein excess (Dickinson and Kohwi-Shigematsu, 1995). In contrast, these proteins exhibited dramatically reduced binding affinity, by at least 2 orders of magnitude, to the mutated $(24)_8$ (Fig, 3A, bottom panel). This indicates that HMG I(Y) has a remarkable specificity toward the wild-type $(25)_7$ probe as opposed to the mutated $(24)_8$ probe, even though these probes are both A+T-rich (see Material and Methods).

To further examine whether it is a general phenomena that HMG I(Y) selectively binds BUR, a gel-mobility shift assay was performed using the native MAR of 300 bp located 3' of the enhancer and the mutated version in which the core unwinding element, ATATAT, is specifically mutated to CTGTCT (Fig. 3B). The 300 bp MAR 3' of the IgH enhancer is 70% A+T rich and the three nucleotide mutations do not significantly alter the overall AT content, and yet the mutation completely abrogates the unwinding property of this MAR (10). As shown in Figure 3B, GST-HMG Y specifically bound wild-type IgH 3'MAR, but not the mutated IgH 3' MAR. Similar results were also found for GST-HMG I (data not shown). Russnak *et al.* (63) has shown that, under the appropriate conditions, HMG-I exhibits different affinities to some A+T rich sequences. Consistent with the previous report that HMG I(Y) strongly interacts with SARs/MARs (20), our

data clearly demonstrated again that HMG I(Y) is not just a nonspecific A+T-rich DNA binding protein but has a binding specificity for BUR.

HMG I(Y) Expression Is Increased in MCF-7 Cells upon Estrogen Treatment or Heregulin cDNA Transfection. Estrogen is known to increase cellular proliferation and activation of estrogen responsive genes in MCF-7 cells, an estrogen receptor (ER) positive cell line (64). Because HMG I(Y) is expressed at elevated levels in proliferating and undifferentiated cells (62), it was of interest to determine if estrogen had any effect on HMG I(Y) expression. Holth *et al.* (41) have shown that estrogen has no significant effect on upregulating HMG I(Y) expression determined by Northern blot analysis. However, by Southwestern and Western analysis, we observed a small induction of MAR-binding activity of HMG I(Y) in MCF-7 cells when they were exposed to estradiol (Figs. 4A and 4C). Two anti-estrogen drugs, Tamoxifen (Tam) and anti-estrogen ICI 164,384 (ICI), completely abolished this induction, indicating that estrogen stimulation of MAR-binding activity was mediated through the ER pathway (Fig. 4A).

HRG is a growth factor expressed in about 30% of invasive breast cancers, and it activates the *erb*B receptor pathway (reviewed in 61). As shown in Table 1, HRG expression correlates with the invasive and metastatic phenotypes of breast cancer cells *in vitro* and *in vivo* (50). HRG is inversely correlated with ER expression. To study the role of HRG in breast cancer tumor progression, Tang *et al.* (53) developed a breast cancer progression model by transfecting an HRG expression construct to an ER positive cell line, MCF-7. MCF-7/HRG-transfected cell clones, which expressed a relatively high level of HRG, developed estrogen independence and resistance to anti-estrogen drugs *in vitro* and *in vivo* (53, 65). This is consistent with a more aggressive hormone-independent phenotype. Wild-type MCF-7 cells are known to be non-tumorigenic in the absence of estrogen and these cells never metastasize in nude mice, even in the presence of estrogen. However, all MCF-7/HRG clones having similar proliferation rates, for example T2, T6, and T7, can form large tumors in nude mice in the absence of an estrogen supplement (65). Tumors generated from T2 and T7 cells (but not T6) became metastatic and developed lymphatic invasion (65). The MCF-7/HRG cell series, therefore, provides a system to study the aggressive

tumor progression in breast cancer. Southwestern and Western analysis of HMG I(Y) were performed for T2, T6, T7, and control MCF-7 cells transfected with vector only. As expected, two metastatic clones, T2 and T7, had significantly increased BUR-binding activity (Fig. 4B) attributed to the elevated level of HMG I(Y) protein (Fig. 4C). In contrast, MCF-7 cells treated with estrogen and T6 cells expressed the same low level of HMG I(Y) as the control MCF-7 cells (Fig. 4C).

Previously, HMG I(Y) expression was shown to be induced by EGF (epidermal growth factor) in metastatic Hs578T cells but not in non-metastatic MCF-7 cells (41). Without induction, no difference of HMG I(Y) mRNA was detected between Hs578T and MCF-7 cells by primer extension and Northern blot analysis (41). However, our Southwestern (Fig. 1) and Western analyses (Fig. 2C) clearly showed that the protein level and BUR-binding activity of HMG I(Y) are both remarkably higher in metastatic cell lines (including Hs578T) than in non-metastatic cell lines (including MCF-7) (see Fig. 2C). Based on our data, there is an association between HMG I(Y) protein level, expression of HRG and malignant phenotype of human breast cancer cells.

It is noteworthy that the protein level and BUR-binding activity of HMG I(Y) in T2 or T7 cells that can metastasize are still much lower than the activity in MDA-MB-231 cells (Fig. 4B). Levels of HMG I(Y) vary dramatically between cell types; for instance, three metastatic cell lines, Hs578T, BT-549, and MDA-MB-231, express different levels of HMG I(Y) even under similar culture conditions (Fig. 1 and Fig. 2C). Therefore, an absolute amount of HMG I(Y) protein or its DNA-binding activity, *per se*, is apparently not the determinant factor for metastasis. However, in a given cell line, the level of HMG I(Y) consistently increases as it becomes more aggressive (due to overexpression of HRG, for example), and decreases as it becomes less aggressive (reduction of HRG expression, see below).

HMG I(Y) Expression Is Reduced in MDA-MB-231 Cells Transfected with a Heregulin Antisense Construct. Since MDA-MB-231 cells are highly metastatic and express high levels of heregulin (50, 53), we studied the effect of reducing the heregulin levels on HMG I(Y) expression by stably transfecting these cells with antisense HRG construct. A series of

stably transfected MDA-MB-231 cell clones were prepared. Cells harboring pRV/CMV vector only (a control called AS-V), and three other cell clones (AS-C6, AS-C23, and AS-C31) transfected with an anti-sense HRG construct showing much reduced levels of HRG expression, were isolated for further analysis. These MDA-MB-231 cells transfected with heregulin antisense HRG-construct have lost metastasizing ability in nude mice (54). Western blot analysis using anti-HMG I(Y) antibody has shown that HMG I(Y) protein levels in all three antisense-HRG cells, AS-C6, AS-C23, and AS-C31, were either dramatically decreased or undetectable (Fig. 5A). This result suggests that the HMG I(Y) level decreased concomitantly with the loss of metastatic phenotype due to the disruption of HRG expression. This result is consistent with our previous results obtained from the studies with MCF-7/HRG cells (Fig. 4C) that the metastatic potential of breast cancer cells is associated with elevated HRG and HMG I(Y) levels.

Treatment of MDA-MB-231 Cells with a Chemical Inhibitor for MMP-9 Reduced the Levels of HMG I(Y). It is generally believed that one key element of the metastatic process is the enhanced proteolysis of both basement membrane and stromal extracellular matrix (ECM). Among the proteinase capable of degrading these barriers are the matrix metalloproteinases (MMPs). The gelatinase A (MMP-2) and B (MMP-9) are two members of the MMP family which have been implicated in breast cancer tumor progression (55, 56). MDA-MB-231 cells appear to secrete a significantly high level of MMP-9 activity as compared with MCF-7 cells (R. Lupu, unpublished result). Inhibition of MMP-9 expression using a ribozyme has been shown to block metastasis in the rat sarcoma model system (57). Consistently, confluent MDA-MB-231 cells treated with a chemical inhibitor for MMP-9, N-methyl-(3S)-2-[(2R)-2-hydroxyaminocarbonylmethyl-1-oxoundecyl]hexahydropyridazine-3-carboxamide in the range of 0.1 to 20 μM concentration, resulted in a significant reduction in the invasive phenotype *in vitro* determined by the Boyden chamber assay (Fig. 5B). At a 10 μM concentration of the inhibitor, the effect became saturated and exhibited approximately 70% inhibition of the invasive activity. The morphology of MDA-MB-231 cells changed from stellar-like to steroid-like shape after treatment

with the MMP-9 inhibitor as revealed by the matrigel outgrowth assay (R. Lupu, unpublished result). The chemical inhibitor used in this study has an IC_{50} value of 38 nM for gelatinase A and an IC_{50} value of 1.2 nM for gelatinase B, and therefore has a high specificity for the latter (66). To examine the effect of the MMP-9 inhibitor on HMG I(Y) expression in MDA-MB-231 cells, these cells in culture at different densities were treated with this MMP-9 specific inhibitor (see Materials and Methods), and their HMG I(Y) expression was measured by Southwestern and Western blot analyses. When subconfluent cells were treated with the MMP-9 inhibitor for 6 days at varying concentrations (1, 2.5, 5 and 10 μM), no significant changes in the level of HMG I(Y) were detected (data not shown). However, when confluent cells were treated with the MMP-9 inhibitor, we observed the gradual decay of HMG I(Y) with the increasing concentration of the MMP-9 inhibitor (Fig. 5C). The MMP-9 inhibitor treatment (10 μM for six days) reduced HMG I(Y) protein levels down to 30% of the original level based on Western analysis (Fig. 5C). Although Southwestern analysis is not as quantitative as Western blot analysis, both assays independently showed an inverse correlation between the concentration of the MMP-9 inhibitor and expression of HMG I(Y). In parallel. the apoptotic cell death had been quantitated by TUNEL assay, because some of the effect maybe due to induction of apoptosis in cells treated with the MMP-9 inhibitor. However, there is no difference on cell viability observed (data not shown) under the conditions used. These data suggest that an alternation in the extracellular matrix by inhibiting MMP-9 resulted in the down regulation of HMG I(Y) expression. Our results show that, similar to the effects of growth factor such as heregulin, HMG I(Y) expression is sensitive to signaling from ECM.

Discussion

Identification of BUR-binding and Metastasis Associated Proteins as HMG I(Y). We detected proteins of ~20 kDa in aggressive human breast carcinoma cells that bind strongly to BURs with a specialized ATC sequence context with high base-unpairing propensity. Using DNA affinity chromatography, a 20kDa protein was purified and identified as HMG I. Another protein with a slightly smaller mass that also confers similar binding specificity was found to be the splicing variant, HMG Y. We demonstrate that HMG I(Y) specifically recognizes BURs: when mutated at specific sites within BURs to abrogate this propensity, HMG I(Y) binding to BURs was either abolished or greatly reduced. This binding specificity of HMG I(Y) for double-stranded BURs is remarkably similar to that of the previously cloned MAR-binding protein SATB1 (13). SATB1 binds along the minor groove with very little contact with the bases, suggesting that SATB1 recognizes the ATC sequence indirectly through the altered sugar-phosphate backbone structure presumably formed due to unusual DNA context.

There are several reports describing the binding specificity of HMG I(Y) proteins (63, 67-69). These studies have shown that HMG I(Y) proteins preferentially bind to the minor groove of A+T-rich B-form DNA (67,68), and yet these proteins do not bind to all stretches of A+T-rich DNA with equal affinity (63). Some study suggests that structure of DNA, rather than the primary sequence *per se*, is a dominant factor that controls HMG I(Y) binding specificity (69). The present study that demonstrated that HMG I(Y) specifically bind to BURs, the key structural elements of MARs, may have an important implication in cancer. BURs are the *in vivo* targets of a cell type-specific MAR-binding protein (15), and ablation of the MAR-binding protein caused major deregulation of multiple genes (70). Furthermore, our most recent study has shown that at least one such BUR is an origin of replication in the human tissue culture system. Not limited to the case of HMG I(Y), but an increase in another BUR-binding protein for more aggressive breast cancer was previously reported (18). BURs could be important genomic loci at which many

regulatory events may take place. Any alternation at these loci, including the level of binding proteins, could lead to malignancy or is necessary to maintain the malignant phenotype.

Association Between HMG I(Y) Expression and Malignant Phenotype in Human Breast Tumor Cells. We examined the changes in HMG I(Y) levels using directly comparable systems using the same cell lines that either express or do not express heregulin. Specifically, we compared non-metastatic MCF-7 cells with MCF-7 cells containing an HRG cDNA construct that became metastatic, and metastatic MDA-MB-231 cells with MDA-MB-231 cells containing an antisense HRG construct that became non-metastatic. This series of experiments allowed us to systematically examine how HMG I(Y) expression varies with malignant progression in human breast cancer cells. Our results were consistent: heregulin overexpression that promoted metastasizing ability resulted in an increase of HMG I(Y) expression, and conversely the reduction of heregulin that blocked metastasis led to a reduction in HMG I(Y) expression. MDA-MB-231/antisense HRG cells have a similar growth rate on plastic culture as compared to vector control cells (54), suggesting that the net expression of HMG I(Y) is not merely a factor of proliferating activity of cells. This demonstrates that there is a direct correlation between an increased HMG I(Y) protein level and high heregulin expression that is linked to metastatic potential in human breast cancer cells, independent of their relative rates of cell proliferation, at least under the experimental condition employed.

We also examined the effect of an MMP-9 chemical inhibitor on the expression of HMG I(Y) in MDA-MB-231 cells. The gelatinase B (MMP-9) is a member of the matrix metalloproteinases (MMPs) which are zinc dependent endopeptidases implicated in cancer invasion and metastasis (56). MMP-9 is known to cleave native collagens of type IV, V and XI, and elastin (reviewed in 71). Blocking MMP-9 activity in MDA-MB-231 cells with the MMP-9 chemical inhibitor resulted in a loss of invasive phenotype *in vitro*, and a decrease of the HMG I(Y) protein levels. The results indicate that disrupting MMP-9 in the extracellular matrix reduced the expression of HMG I(Y). Blocking function of ECM proteinases can decrease tumor growth (57,

72, 73). Therefore, it is conceivable that a specific signalling pathway(s) is affected due to the MMP-9 inhibitor such that cancer cells become less aggressive as evidenced by the result of Boyden Chamber assay (Fig. 5B). HMG I(Y) expression may be a downstream event and decrease in its expression might be one of the nuclear responses to ECM modification.

Based on our results, it appears that the absolute amount of HMG I(Y) is not a determining factor for the metastatic phenotype. For example, MMP-9 inhibitor treated cells still retained a high level of HMG I(Y), although it was reduced in comparison to the original levels. Therefore, changes in the level of HMGI(Y) expression appear to correlate with or reflect the changes in the status of malignancy of given breast carcinoma cells.

Potential Role of HMG I(Y) in Breast Cancer. Transcriptional control has become a major focus in current cancer research. Transcription of the HMG I(Y) gene is predicted to be tightly regulated, and has been shown specifically to be induced in human cells by phorbol esters (74), calcium ionophores (75) and EGF (41), and upregulated by human papillomavirus type 16 E6 protein (76) and tumor promoter (28) in mouse cells. The transcriptional regulation and functional role of HMG I(Y) in cancer development are, however, essentially unknown.

Several functions for HMG I(Y) proteins have been proposed, including nucleosome phasing (77), involvement in the 3' end processing of mRNA transcripts (63, 78), and the amplification of autonomously replicating sequences (79). HMG I(Y) proteins have been demonstrated to be involved in both positive and negative regulation of genes containing A+T-rich regions of DNA, possibly by functioning as accessory "architectural transcription factors" (80-86). HMG I(Y) proteins also appear to bind specifically to regions of putative mammalian cell origins of replication (87) and to G/Q and C-bands of metaphase chromosomes (88). More recently, three distinct sub-nuclear populations of HMG I(Y) have been demonstrated (89), suggesting they may be required for chromosome structural change during cell cycle. An increase in HMG I(Y) gene expression associated with progression in the malignant phenotype may have effects on any combination of these molecular interactions.

The nuclear matrix is believed to play critical roles in regulating many key biological reactions in the nucleus such as gene transcription, DNA replication, DNA organization, and RNA splicing and processing (reviewed in 90). Our data suggest that HMG I(Y) interacts with BURs, the key structural element of MARs. HMG I(Y) may assemble specific three dimensional transcription and/or replication complexes at BUR sites. If so, this may cause detachment of the sites from the nuclear matrix, by successfully competing BURs sites from other MAR-binding proteins, and subsequently alter the loop domain structure of chromatin. It would be interesting to explore this possibility in the future.

Acknowledgments

This work was supported by the National Institutes of Health (RO3 CA70824 to T. Kohwi-Shigematsu) and the U.S. Department of Energy under contract No. DE-AC03-76SF00098; and by the U.S. Army BCRP DAMD 17-98-1-8303 to W.-M. Liu, the RO1-DK to R. Lupu and the UCBCRP to F. Guerra-Vladusic. This work was also supported by Sankyo Co., Ltd. which provided equipment funds. The authors thank our colleagues, Drs. Sanjeev Galande and Miaw-Sheue Tsai, for technical assistance and valuable discussion.

Figure Legend

Figure 1: The high level of MAR-binding activity due to small molecular-weight proteins (around 20 kDa) occurs in human metastatic breast carcinoma cells (lane 5 - lane 7) but not in non-metastatic cells (lane 1 - lane 4). Southwestern blot analysis was performed to investigate MAR-binding activity. Twenty five μg of proteins in cell extracts were loaded on a 15% SDS-polyacrylamide gel. After electrophoresis, the proteins were renatured, then the gel was blotted and hybridized with the radiolabeled wild-type $(25)_7$ probe. The same amount of proteins used in the Southwestern blot was subjected to Western blot analysis using anti-β-actin antibody.

Table 1: Non-metastatic vs. metastatic human breast cancer cell lines

(NT: Non Tumorigenic)

Figure 2: The 20 kDa protein was identified as HMG I(Y). (**A**) Affinity purification of a MAR-binding protein from a breast carcinoma cell line, MDA-MB-231. Commassie blue staining analysis was performed for 40 μg protein of a crude cell extract prepared from the MDA-MB-231 cells (Crude) and column-purified p20 from the MDA-MB-231 cell extract (Purified). M, molecular size marker. Arrowhead, p20. (**B**) Southwestern blot analysis using radiolabeled wild-type $(25)_7$ as the probe was performed with the same crude extract and purified fraction (1/100) from (A). (**C**) Western blot analysis using anti-HMG I(Y) antibody was performed for 25 μg protein of cell extracts prepared from human breast carcinoma cell lines. M, molecular size marker. Top panel, shorter exposure. Bottom panel, longer exposure.

Figure 3: Gel-mobility shift assay was performed for GST-HMG I and GST-HMG Y with a radiolabeled wild-type $(25)_7$ probe (**A**, top panel), a mutated $(24)_8$ probe (**A**, bottom panel), a wild-

type or a mutated IgH 3'MAR probe (**B**, left and right). The DNA probes were incubated with varying amounts of protein in 20 μl of binding reaction mixture and electrophoresed on a 6% native polyacrylamide gel. Protein concentrations are indicated in [nM].

Figure 4: HMG I(Y) expression is increased in MCF-7 cells upon estrogen treatment or heregulin cDNA transfection. Southwestern blot analysis using radiolabeled wild-type $(25)_7$ as the probe was performed with (A) and (B). Western blot analysis of HMG I(Y) was performed with (C). (**A**) Twenty five μg of proteins extracted from MCF-7 and MDA-MB 231 cells with various treatments for 48 hours were used for Southwestern analysis. C, control; E_2, estradiol (10^{-9}M); ICI, anti-estrogen ICI 164,384 (10^{-7}M); Tam, Tamoxifen (10^{-7}M). (**B**) Twenty five μg of proteins extracted from MCF-7 and MDA-MB-231 cells were used for Southwestern analysis. MCF-7 cells transfected with the pRC/CMV vector only are indicated as V in left upper panel and as MCF-7/HRG-V in the right upper panel. Three MCF-7 clones transfected with heregulin-β2 cDNA are indicated as T_2, T_6, and T_7. The same amount of protein used in the Southwestern blot was subjected to Western blot analysis using anti-β-actin antibody. (**C**) Fifty μg proteins extracted from MCF-7 cells, MCF-7 cells treated with E_2, and MCF-7/T cells were used in the Western blot analysis using anti-HMG I(Y) antibody.

Figure 5: HMG I(Y) expression is reduced in MDA-MB-231 cells upon antisense-HRG transfection and MMP-9 inhibitor treatment. (**A**) Twenty μg of proteins extracted from MDA-MB-231/AS serious cells were used for Western blot analysis of HMG I(Y) and β-actin. MDA-MB-231 cells transfected with the pRC/CMV vector only are indicated as V. Three MDA-MB-231/AS clones transfected with antisense-HRG are indicated as C6, C23, and C31. (**B**) Boyden chamber assay: MDA-MB-231 cells were tested in the Boyden chamber assay in the presence or absence of

increasing concentrations (0-20 μM) of the MMP-9 inhibitor for 6 hours. Chemoinvasion was measured according to the number of cells transversing matrigel-coated filters. Data points present the mean from quadruplicate from a representative experiment. Standard deviation was calculated for each data point. (**C**) Twenty μg of proteins extracted from MDA-MB-231 cells with MMP-9 inhibitor treatments for 6 days at indicated concentration (0, 2.5, 5, and 10 μM) were used for Southwestern analysis using a radiolabeled wild-type $(25)_7$ probe, and Western blot analysis of HMG I(Y) and β-actin. Quantitation of Southwestern blot and Western blot were done individually by phosphorimager and densitometer.

References

1. Mirkovitch, J., Mirault, M.-E., and Laemmli, U. K. Organization of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold. Cell, *9:* 223-232, 1984.
2. Cockerill, P. N., and Garrard, W. T. Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerases II sites. Cell, *44:* 273-282, 1986.
3. Gasser, S. M., and Laemmli, U. K. Cohabitation of scaffold binding regions with upstream/enhancer elements of three developmentally regulated genes of *D. melanogaster.* Cell, *46:* 521-530, 1986.
4. Gasser, S. M., and Laemmli, U. K. The organization of chromatin loops: characterization of a scaffold. EMBO J., *5:* 511-518, 1986.
5. Cockerill, P. N., Yuen, M.-H., and Garrard, W. T. The enhancer of the immunoglobulin heavy chain locus is flanked by presumptive chromosomal loop anchorage elements. J Biol. Chem., *262:* 5394-5397, 1987.
6. Gasser, S. M., and Laemmli, U. K. A glimpse at chromosomal order. Trends Genet., *3:* 16-22, 1987.
7. Forrester, W. C., van Genderen, C., Jenuwein, T., and Grosschedl, R. Dependence of enhancer-mediated transcription of the immunoglobulin μ gene on nuclear matrix attachment regions. Science, *265:* 1221-1225, 1994.
8. Kirillov, A., Kistler, B., Mostsolasky, R., Cedar, H., Wirth, T. and Bergman, Y. A role for nuclear NF-κB-cell-specific demethylation of the *Igk* locus. Nat. Genet., *13:* 435-441, 1996.
9. Jenuwein, T., Foresster, W. C., Fernandez-Herrero, L. A., Laible, G., Dull, M., and Grosschedl, R. Extension of chromatin accessibility by nuclear matrix attachment regions. Nature, *385:* 269-272, 1997.

10. Kohwi-Shigematsu, T., and Kohwi, Y. Torsional stress stabilizes extended base unpairing in suppressor sites flanking immunoglobulin heavy chain enhancer. Biochemistry, *29:* 9551-9560, 1990.

11. Bode, J., Kohwi, Y., Dickinson, L. A., Joh, T., Klehr, D., Mielke, C., and Kohwi-Shigematsu, T. Biological significance of unwinding capability of nuclear matrix-associating DNAs. Science, *255:* 195-197, 1992.

12. Kohwi-Shigematsu, T., and Kohwi, Y. *In:* R. Curtis Bird (ed.) Nuclear Structure and Gene Expression: High unwinding capability of matrix attachment regions and ATC-sequence context-specific MAR-binding proteins, pp. 111-144. San Diego: Academic Press, 1997.

13. Dickinson, L. A., Joh, T., Kohwi, Y., and Kohwi-Shigematsu, T. A tissue-specific MAR/SAR DNA-binding protein with unusual binding site recognition. Cell, *70:* 631-645, 1992.

14. Herrscher, R. F., Kaplan, M. H., Lelsz, D. L., Das, C., Scheuermann, R., and Tucker, P. W. The immunoglobulin heavy-chain matrix-associating regions are bound by Bright: a B cell-specific trans-activator that describes a new DNA-binding protein family. Genes Dev., *9:* 3067-3082, 1995.

15. de Belle, I., Cai, S., and Kohwi-Shigematsu, T. The genomic sequences bound to special AT-rich sequence-binding protein 1 (SATB1) in vivo in Jurkat T cells are tightly associated with the nuclear matrix at the bases of the chromatin loops. J. Cell Biol., *141:* 335-348, 1998.

16. Dickinson, L. A., and Kohwi-Shigematsu, T. Nucleolin is a matrix attachment region DNA-binding protein that specifically recognizes a region with high base-unpairing potential. Mol. Cell Biol., *15:* 456-465, 1995.

17. Will, K., Warnecke, G., Wiesmuller, L., and Deppert. W. Specific interaction of mutant p53 with regions of matrix attachment region DNA elements (MARs) with a high potential for base-unpairing. Proc. Natl. Acad. Sci. USA, *95:* 13681-13686, 1998.

18. Yanagisawa, J., Ando, J., Nakayama, J., Kohwi, Y., and Kohwi-Shigematsu, T. A matrix attachment region (MAR)-binding activity due to a p114 kilodalton protein is found only in

human breast carcinomas and not in normal and benign breast disease tissues. Cancer Res., *56:* 457-462, 1996.

19. Johnson, K. R., Lehn, D. A., and Reeves, R. Alternative processing of mRNA encoding mammalian chromosomal high-mobility-group proteins HMG-I and HMG-Y. Mol. Cell Biol., *9:* 2114-2123, 1989.
20. Zhao, K., Kas, E., Gonzalez, E., and Laemmli, U. K. SAR-dependent mobilization of histone H1 by HMG-I/Y in vitro: HMG-I/Y is enriched in H1-depleted chromatin. EMBO J., *12:* 3237-3247, 1993.
21. Wunderlich, V., and Bottger, M. High -mobility-group proteins and cancer - an emerging link. J. Cancer Res. Clin. Oncol., *123:* 133-140, 1997.
22. Lund, T., Holtlund, J., Fredriksen, M., and Laland, S. G. On the presence of two new high mobility group-like proteins in HeLa S3 cells. FEBS Lett., *152:* 163-167, 1983.
23. Giancotti, V., Berlingieri, M. T., Di Fiore, P. P., Fusco, A., Vecchio, G., and Crane-Robinson, C. Changes in nuclear proteins on transformation of rat epithelial thyroid cells by a murine sacroma retrovirus. Cancer Res., *45:* 6051-6057, 1985.
24. Giancotti, V., Pani, B., D'Andrea, P., Berlingieri, M. T., Di Fiore, P. P., Fusco, A., Vecchio, G., Philp, R., Crane-Robinson, C., Nicolas, R. H., Wright, C. A., and Goodwin, G. H. Elevated levels of a specific class of nuclear phosphoproteins in cells transformed with v-*ras* and v-*mos* oncogenes and by co-transfection with c-*myc* and polyoma middle T genes. EMBO J., *6:* 1981-1987, 1987.
25. Giancotti, V., Buratti, E., Perissin, L., Zorzet, S., Balmain, A., Portella, G., Fusco., A., and Goodwin, G. H. Analysis of the HMGI nuclear proteins in mouse neoplastic cells induced by different procedures. Exp. Cell Res., *184:* 538-545, 1989.
26. Bussemakers, M. J., van de Ven, W. J. M., Debruyne, F. M. J., and Schalken, J. A. Identification of high mobility group protein I(Y) as potential progression marker for prostate cancer by differential hybridization analysis. Cancer Res., *51:* 606-611, 1991.

27. Ram T. G., Reeves, R., and Hosick, H. L. Elevated high mobility group-I(Y) gene expression is associated with progressive transformation of mouse mammary epithelial cells. Cancer Res., *53:* 2655-2660, 1993.

28. Cmarik, J. L., Li, Y., Ogram, S. A., Min, H., Reeves, R., and Colburn, N. H. Tumor promoter induces high mobility group HMG-Y protein expression in transformation-sensitive but not –resistant cells. Oncogene, *16:* 3387-3396, 1998.

29. Berlingieri, M T., Manfioletti, G., Santord, M., Bandiera, A., Visconti, R., Giancotti, V., and Fusco, A. Identification of HMG I-C protein synthesis suppresses retrovially induced neoplastic transformation of rat thyroid cells. Mol. Cell Biol., *15:* 1545-1553, 1995.

30. Chiappetta, G., Tallini, G., De Biasio, M. C., Manfioletti, G., Martinez-Tello, F. J., Pentimalli, F., de Nigris, F., Mastro, A., Botti, G., Fedele, M., Berger, N., Santoro, M., Giancotti, V., Fusco, A. Detection of high mobility group I HMGI(Y) protein in the diagnosis of thyroid tumors: HMGI(Y) expression represents a potential diagnostic indicator of carcinoma. Cancer Res., *58:* 4193-4198, 1998.

31. Tamimi, Y., van der Poel, H. G., Denyn, M.-M., Umbas, R., Karthaus, H. F. M., Debruyne, F. M. J., and Schalken, J. A. Increased expression of high mobility group protein I(Y) in high grade prostatic cancer determined by *in situ* hybridization. Cancer Res., *53:* 5512-5516, 1993.

32. Tamimi, Y., van der Poel, H. G., Karthaus, H. F., Debruyne, M., and Schalken, J. A retrospective study of high mobility group protein I(Y) as progression marker for prostate cancer determined by in situ hybridization. Br. J. Cancer, *74:* 573-578, 1996.

33. Fedele, M., Bandiera, A., Chiappetta, G., Battista, S., Vigletto, G., Manfioletti, G., Gasamassimi, A., Santoro, M., Giancotti, W., and Fusco, A. Human colorectal carcinomas express high levels of high mobility group HMGI(Y) proteins. Cancer Res., *56:* 1896-1901, 1996.

34. Williams, A. J., Powell, W. L., Collins, T., and Morton C. C. HMGI(Y) expression in human uterine leiomyomata. Amer. J. Pathol., *150:* 911-918, 1997.

35. Bandiera, A., Bonifacio, D. M. T., Manfioletti, G., Mantovani, F., Rustighi, A., Zanconati, F., Fusco, A., Bonito, L. D., and Giancotti, V. Expression of HMGI(Y) proteins in squamous intraepithelial and invasive lesions of the uterine cervix. Cancer Res., *58:* 426-431, 1998.

36. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science, *235*: 177-182, 1987.

37. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., and Ullrich, A. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science, *244*: 707-712, 1989.

38. Harris, J. R., Lippman, M. E., Veronesi, U., and Willett, W. Breast cancer (3). New Eng. J. Med., *327*: 473-480, 1992.

39. Dougall, W. C., Qian, X., Peterson, N. C., Miller, M. J., Samanta, A., and Greene, M. I. The neu-oncogene: signal transduction pathways, transformation mechanisms and evolving therapies. Oncogene, *9*: 2109-2123, 1994.

40. Klijn, J. G., Berns, P. M., Schmitz, P. I., and Foekens, J. A. The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. Endo. Rev., *13*: 3-17, 1992.

41. Holth, L. T., Thorlacius, A. E., and Reeves, R. Effects of epidermal growth factor and estrogen on the regulation of the HMG-I/Y gene in human mammary epithelial cell lines. DNA Cell Biol., *16:* 1299-1309, 1997.

42. van de Vijver, M. J., Peterse, J. L., Mooi, W. J., Wisman, P., Lomans, J., Dalesio, O., and Nusse, R. Neu-protein overexpression in breast cancer. Association with comedo-type ductal carcinoma in situ and limited prognostic value in stage II breast cancer. New Eng. J. Med., *319*: 1239-1245, 1988.

43. Lacroix, H., Iglehart, J. D., Skinner, M. A., and Kraus, M. H. Overexpression of erbB-2 or EGF receptor proteins present in early stage mammary carcinoma is detected simultaneously in matched primary tumors and regional metastases. Oncogene, *4*: 145-151, 1989.

44. de Potter, C. R., Beghin, C., Makar, A. P., Vandekerckhove, D., and Roels, H. J. The neu-oncogene protein as a predictive factor for haematogenous metastases in breast cancer patients. Int. J. Cancer, *45*: 55-58, 1990.

45. Paik, S., Hazan, R., Fisher, E. R., Sass, R. E., Fisher, B., Redmond, C., Schlessinger, J., Lippman, M. E., and King, C. R. Pathologic findings from the National Surgical Adjuvant Breast and Bowel Project: prognostic significance of erbB-2 protein overexpression in primary breast cancer. J. Clin. Oncol., *8*: 103-112, 1990.

46. Allred, D. C., Clark, G. M., Molina, R., Tandon, A. K., Schnitt, S. J., Gilchrist, K. W., Osborne, C. K., Tormey, D. C., and McGuire, W. L. Overexpression of HER-2/neu and its relationship with other prognostic factors change during the progression of in situ to invasive breast cancer. Hum. Pathol., *23*: 974-979, 1992.

47. Gusterson, B. A., Gelber, R. D., Goldhirsch, A., Price, K. N., Säve-Söderborgh, J., Anbazhagan, R., Styles, J., Rudenstam, C. M., Golouh, R., and Reed, R. Prognostic importance of c-erbB-2 expression in breast cancer. International (Ludwig) Breast Cancer Study Group. J. Clin. Oncol., *10*: 1049-1056, 1992.

48. Toikkanen, S., Helin, H., Isola, J., and Joensuu, H. Prognostic significance of HER-2 oncoprotein expression in breast cancer: a 30-year follow-up. J. Clin. Oncol., *10*: 1044-1048, 1992.

49. Lupu, R., Colomer, R., Zugmaier, G., Sarup, J., Shepard, M., Slamon, D., and Lippman M. E. Direct interaction of a ligand for thre *erb*B-2 oncogene product with the EGF receptor and $p185^{erbB-2}$. Science, *249:* 1552-1555, 1990.

50. Staebler, A., Sommers, C., Mueller, S., Byers, S., Thompson, E. W., and Lupu, R. Modulation of breast cancer progression and differentiation by the gp30/heregulin. Breast Cancer Res. Treat., *31:* 175-182, 1994.

51. Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Reeb, H., and Lewis, G. D. Identification of HRGs, a specific activator of $p185^{erbB-2}$. Science, *256:* 1206-1210, 1992.

52. Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Levy, R. B., and Yarden, Y. Isolation of the Neu/HER-2 stimulatory ligand: a 44 kDa glycoprotein that induces differentiation of mammary tumor cells. Cell, *69:* 205-216, 1992.

53. Tang, C., Perez, C., Grunt, T., Waibel, C., Cho, C., and Lupu, R. Involvement of heregulin-β2 in the acquisition of the hormone-independent phenotype of breast cancer cells. Cancer Res., *56*: 3350-3358, 1996.

54. Shamon, L., Tang, C.K., Cardillo, M., Vladusic, E.A., and Lupu, R. manuscript in preparation

55. Collier, I. E., Wilhelm, S. M., Eisen, A. Z., Marmer, B. I., Grant, G. A., Seltzer, J. I., Kronberger, A., He, C. S., Bauer, E. A., and Goldgerg, G. I. H-*ras* oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloprotease capable of degrading basement membrane collagen. J. Biol. Chem., *263:* 6579-6587, 1988.

56. Wilhelm, S. M., Collier, I. E., Marmer, B. I., Eisen, A. Z., Grant, G. A., and Goldgerg, G. I. SV-40 transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. J. Biol. Chem., *264:* 17213-17221, 1989.

57. Hua, J., and Muschel, R. J. Inhibition of matrix metalloproteinase 9 expression by a ribozyme blocks metastasis in a rat sarcoma model system. Cancer Res., *56:* 5279-5284,

58. Thompson, E. W., Katz, D., Shima, T. B., Wakeling, A. E., Lippman, M. E., and Dickson, R. B. ICI 164,384, a pure antagonist of estrogen-stimulated MCF-7 cell proliferation and invasiveness. Cancer Res., *49:* 6929-6934, 1989.

59. Dickinson, L. A., Dickinson, C. D., and Kohwi-Shigematsu, T. An atypical homeodomain in SATB1 promotes specific recognition of the key structural element in a matrix attachment region. J. Biol. Chem., *272*: 11463-11470, 1997.

60. Lupu, R., Dickson, R. B., and Lippman, M. E. The role of *erb*B-2 and its ligands in growth control of malignant breast epithelium. Princess Takamatsu Symp., *22*: 49-60, 1991.

61. Lupu, R., Cardillo, M., Cho, C., Harris, L., Hijazi, M., Perez, C., Rosenberg, K., Yang, D., and Tang, C. The significance of heregulin in breast cancer tumor progression and drug resistance. Breast Cancer Res. Treat., *38:* 57-66, 1996.

62. Johnson, K. R., Disney, J. E., Wyatt, C. R., and Reeves, R. Expression of mRNAs encoding mammalian chromosomal proteins HMG-I and HMG-Y during cellular proliferation. Exp. Cell Res., *187:* 69-76, 1990.

63. Russnak, R. H., Candido, E. P. M., and Astell, C. R. Interaction of the mouse chromosomal protein HMG-I with the 3' ends of genes *in vitro*. J. Biol. Chem., *263:* 6392-6399, 1988.

64. Pratt, S. E., and Pollak, M. N. Estrogen and antiestrogen modulation of MCF-7 human breast cancer cell proliferation is associated with specific alterations in accumulation of insulin-like growth factor-binding proteins in conditioned media. Cancer Res., *53:* 5193-5198, 1993.

65. Cardillo, M., Tang, C. K., and Lupu, R. Abstract published in the American Association for Cancer Research Meeting, March 1995, Toronto Canada.

66. Tamaki, K., Tanzawa, K., Kurihara, S., Oikawa, T., Monma, S., Shimada, K., and Sugimura, Y. Synthesis and structure-activity relationships of gelatinase inhibitors derived from matlystatins. Chem. Pharm. Bulle., *43:* 1883-93, 1995.

67. Reeves, R., and Nissen, M. S. The A-T-DNA-binding domain of mammalian high mobility group I chromosomal proteins. J. Biol. Chem., *265:* 8573-8582, 1990.

68. Wegner, M., and Grummt, F. Netropsin, distamycin and berenil interact differentially with a high-affinity binding site for the high mobility group protein HMG-I. Biochem. Biophys. Res. Commun., *166:* 1110-1117, 1990.

69. Maher, J. F., and Nathans, D. Multivalent DNA-binding properties of the HMG-I proteins. Proc. Natl. Acad. Sci. USA, *93:* 6716-6720, 1996.

70. Alvarez, J. D., Yasui, D. H., Niida, H., Joh, T., Loh, D. Y., and Kohwi-Shigematsu, T. The MAR-binding protein, SATB1, orchestrates temporal and spatial expression of multiple genes during T-cell development. (submitted)

71. Vu, T. H. and Werb, Z. *In:* W. C. Parks and R. P. Mecham (eds.) Gelatinase B: structure, regulation, and function. In Matrix Metalloproteinases, pp. 115-148. San Diego: Academic Press, 1998.

72. Martin, D. C., Rüther, U., Sanchez-Sweatman, O. H., Orr, F. W., and Khokha, R. Inhibition of SV40 T antigen-induced hepatocellular carcinoma in TIMP-1 transgenic mice. Oncogene, *13:* 569-76, 1996.

73. Wilson, C. L., Heppner, K. J., Labosky, P. A., Hogan, B. L., and Matrisian, L. M. Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. Proc. Natl. Acad. Sci.USA., *94:* 1402-7, 1997.

74. Ogram, S. A., and Reeves, R. Differential regulation of a multipromoter gene. J. Biol. Chem., *270:* 14235-14242, 1995.

75. Friedmann, M., Holth, L. T., Zoghbi, H. Y., and Reeves, R. Organization, inducible-expression and chromosome localization of the human HMG-I(Y) nonhistone protein gene. Nucl. Acids Res., *21:* 4259-4267, 1993.

76. Kinoshita, T., Shirasawa, H., Shino, Y., Shimizu, K., Moriya. H., and Simizu, B. Human papillomavirus type 16 E6 protein up-regulates the expression of the high mobility group protein HMG-I(Y) gene in mouse 10T1/2 cells. Virus Res., *42:* 119-125, 1996.

77. Stauss, F., and Varshavsky, A. A protein binds to a satellite DNA repeat at three specific sites that would be brought into mutual proximity by DNA folding in the nucleosome. Cell, *37:* 889-901, 1984.

78. Reeves, R., Elton, T. S., Nissen, M. S., Lehn, D., and Johnson, K. R. Posttranscriptional gene regulation and specific binding of the nonhistone protein HMG-I by the 3' untranslated region of bovine interleukin 2 cDNA. Proc. Natl. Acad. Sci. USA, *84:* 6531-6535, 1987.

79. Wegner, M., Helftenbein, E., Muller, F., Meinecke, M., Muller, S., and Grummt, F. Identification of an amplification promoting DNA sequence from the hypotrichous ciliate *Stylonychis lemnae.* Nucleic Acids Res., *17:* 8783-8802, 1989.

80. Wolffe, A. P. Architectural transcription factors. Science, *264*: 1100-1101, 1994.

81. Grosschedl, R. Higher-order nucleoprotein complexes in transcription: Analogies with site-specific recombination. Curr. Opin. Cell Biol., *7:* 362-370, 1995.

82. Bustin, M., and Reeves, R. High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function. Prog. Nucleic Acid Res. Mole. Biol., *54*: 35-100, 1996.

83. Bagga, R., and Emerson, B. M. An HMG I/Y-containing repressor complex and supercoiled DNA topology are critical for long-range enhancer-dependent transcription *in vitro.* Genes Deve., *11:* 629-639, 1997.

84. Chin, M. T., Pellacani, A., Wang, H., Lin, S. S. J., Jain, M. K., Perrella, M. A., and Lee, M.-E. Enhancement of serum-response factor-dependent transcription and DNA binding by the architectural transcription factor HMG-I(Y). J. Biol. Chem., *273:* 9755-9760, 1998.

85. Bonnefoy, E., Bandu, M.-T., and Doly, J. Specific binding of High-mobility-group I (HMGI) protein and histone H1 to the upstream AT-rich region of the murine beta interferon promoter: HMGI protein acts as a potential antirepressor of the promoter. Mol. Cell Biol., *19:* 2803-2816, 1999.

86. Chase, M. B., Haga, S. B., Hankins, W. D., Williams, D. M., Bi, Z., Strovel, J. W., Obriecht, C., and Berg, P. E. Binding of HMG-I(Y) elicits structural changes in a silencer of the human beta-globin gene. Amer. J. Hematol., *60:* 27-35, 1999.

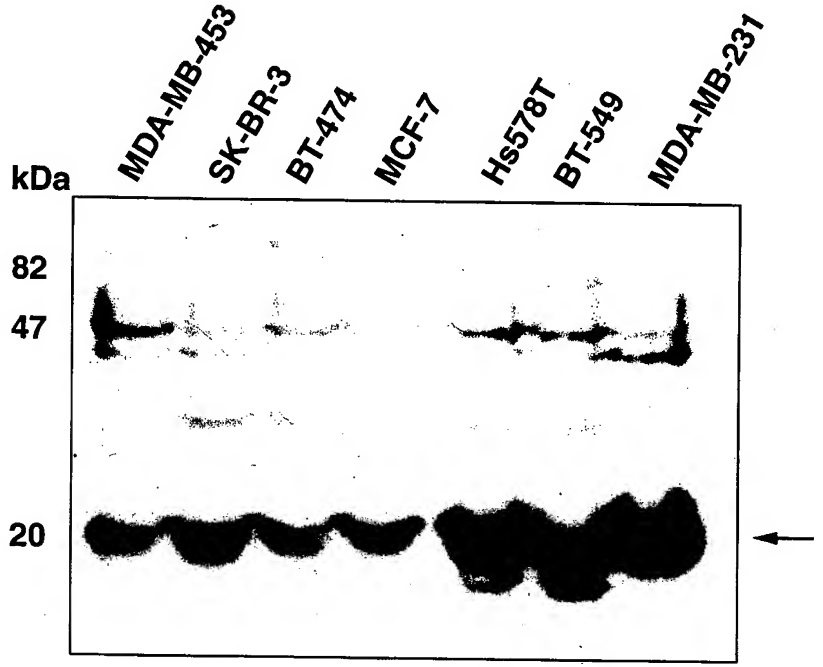
87. Grummt, F., Hoist, A., Muller, F., Wegner, M., Schwender, S., Luksza, H., Zastrow, G., and Klavinius, A. Mouse ARS elements and ARS-binding proteins. Cancer cells (Cold Spring Harbor), *6:* 463-466, 1988.

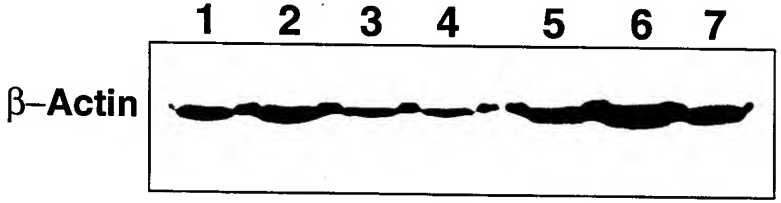
88. Disney, J. E., Johnson, K. R., Magnuson, N. S., Sylvester, S. R., and Reeves, R. High-mobility group protein HMG-I localizes to G/Q-and C-bands of human and mouse chromosomes. J. Cell Biol., *109:* 1975-1982, 1989.

89. Amirand, C., Viari, A., Ballini, J. P., Rezaei, H., Beaujean, N., Jullien, D., Käs, E., and Debey, P. Three distinct sub-nuclear populations of HMG-I protein of different properties revealed by co-localization image analysis. J. Cell Sci., *111:* 3551-3561, 1998.

90. Nelson, W. G., Pienta, K. J., Barrack, E. R., and Coffey, D. S. The role of the nuclear matrix in the organization and function of DNA. Annu. Rev. Biophys. Chem., *15:* 457-475, 1986.

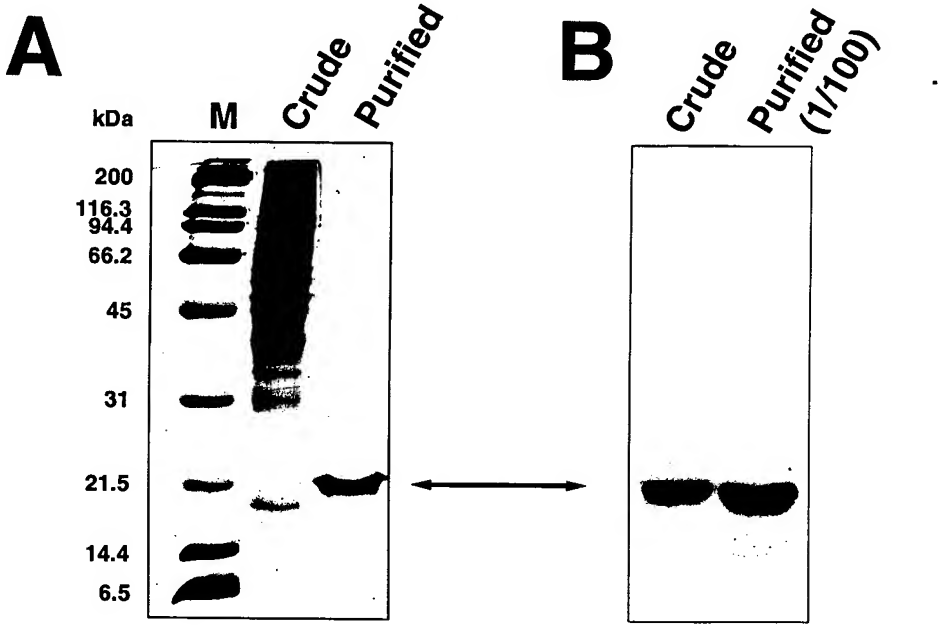
Figure 1





	Cell line	HRG	ER	Vimentin	Invasive In vitro	Metastatic In vivo
1	MDA-MB-453	--	--	--	--	NT
2	SK-BR-3	--	--	--	--	NT
3	BT-474	--	+++	--	--	-1
4	MCF-7	--	++++	--	--	-1
5	Hs578T	++++	--	++++	++++	+
6	BT-549	+++	--	++++	+++	+
7	MDA-MB-231	++++	--	++++	++++	+

Figure 2



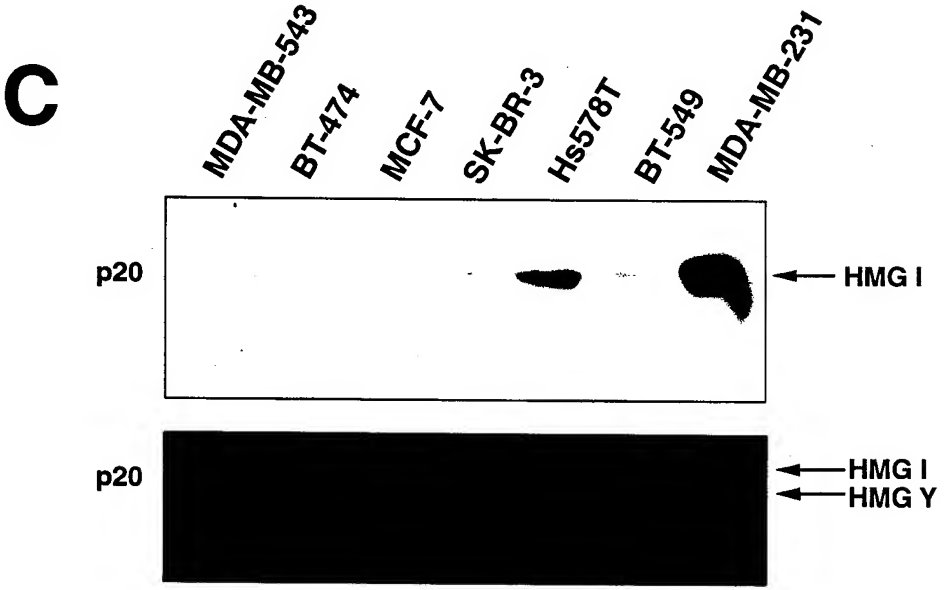
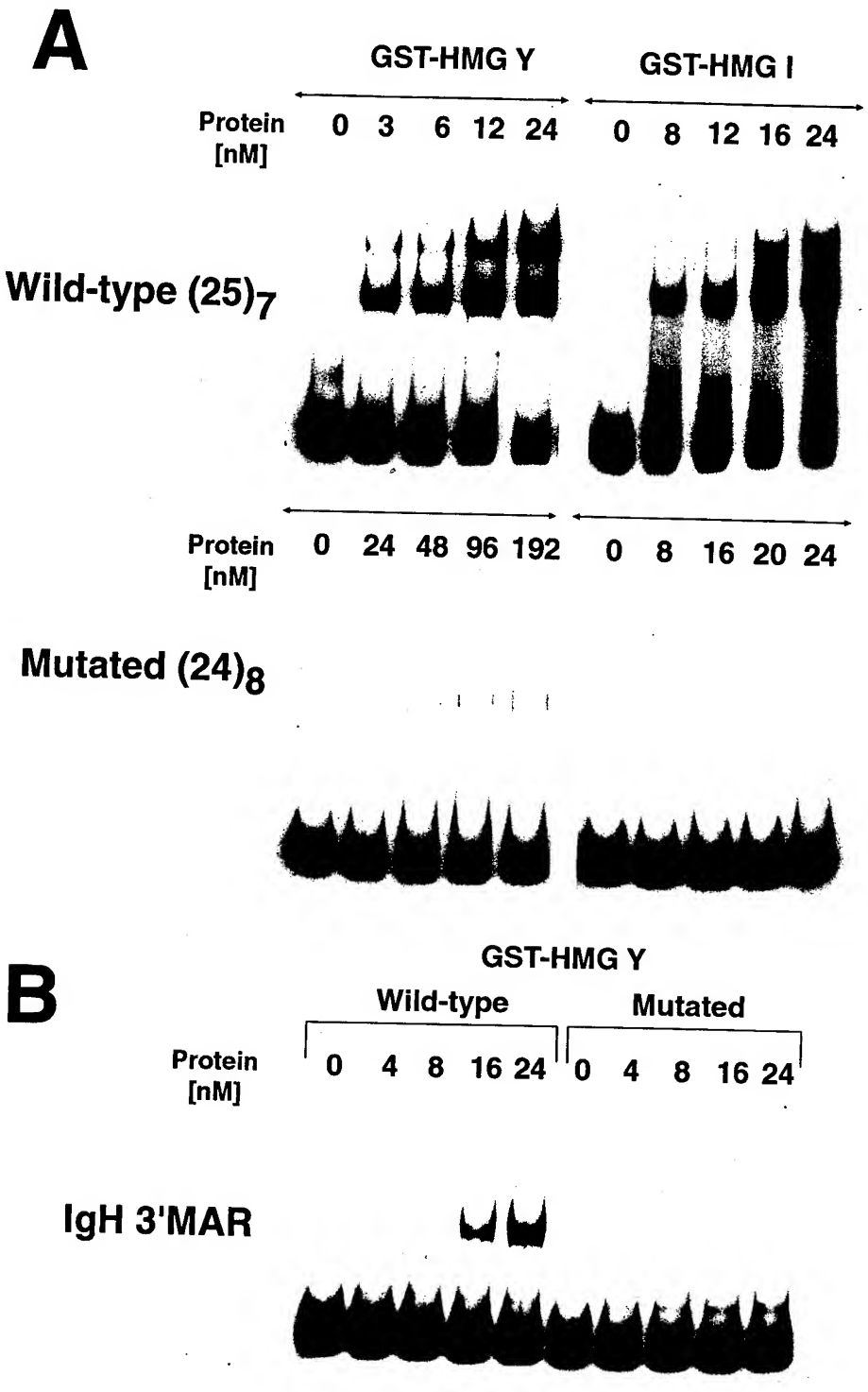


Figure 3


A
GST-HMG Y
GST-HMG I
Protein [nM]
0 3 6 12 24
0 8 12 16 24
Wild-type (25)7
Protein [nM]
0 24 48 96 192
0 8 16 20 24
Mutated (24)8
B
GST-HMG Y
Wild-type
Mutated
Protein [nM]
0 4 8 16 24
0 4 8 16 24
IgH 3'MAR

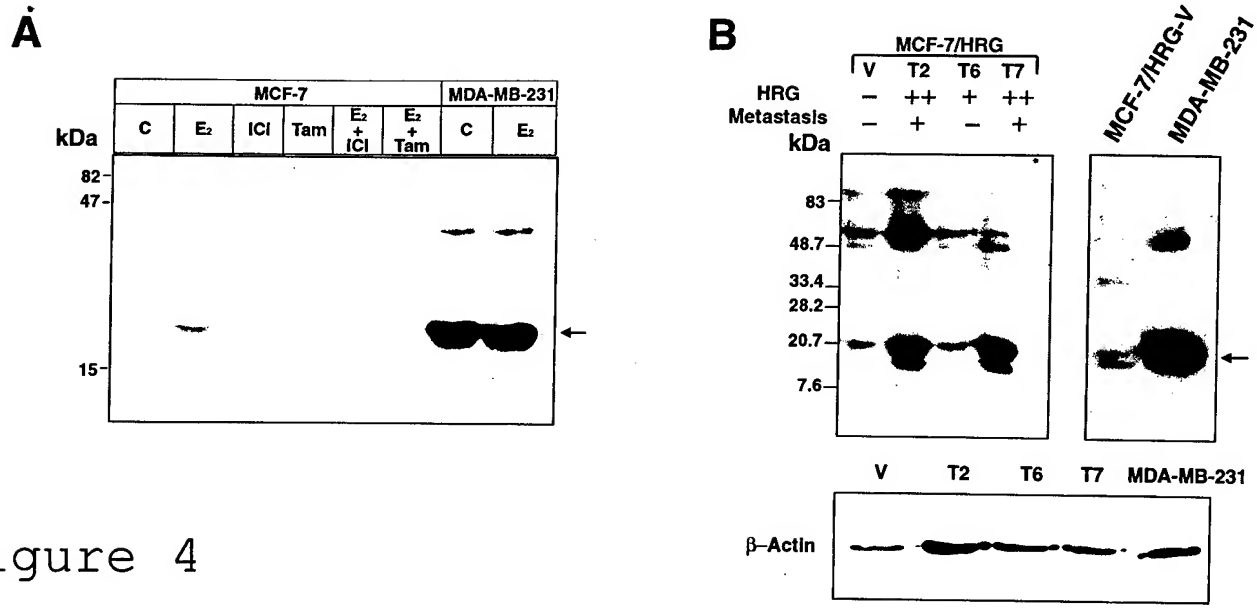
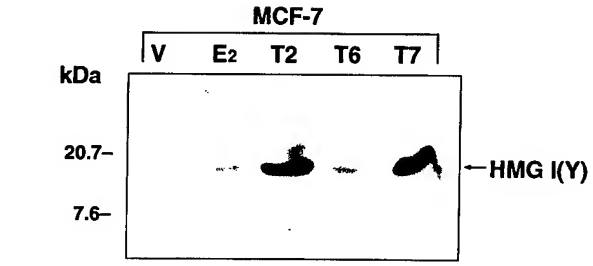
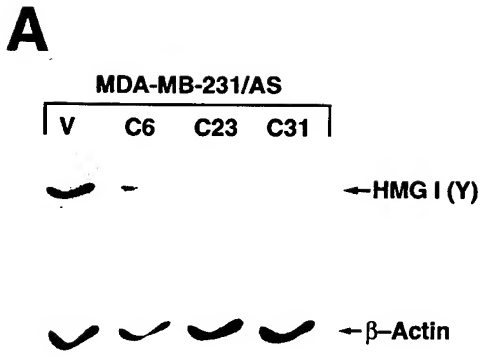


Figure 4





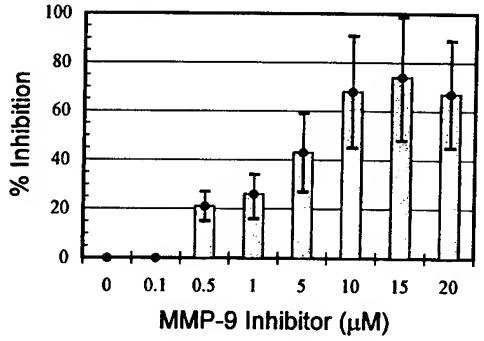
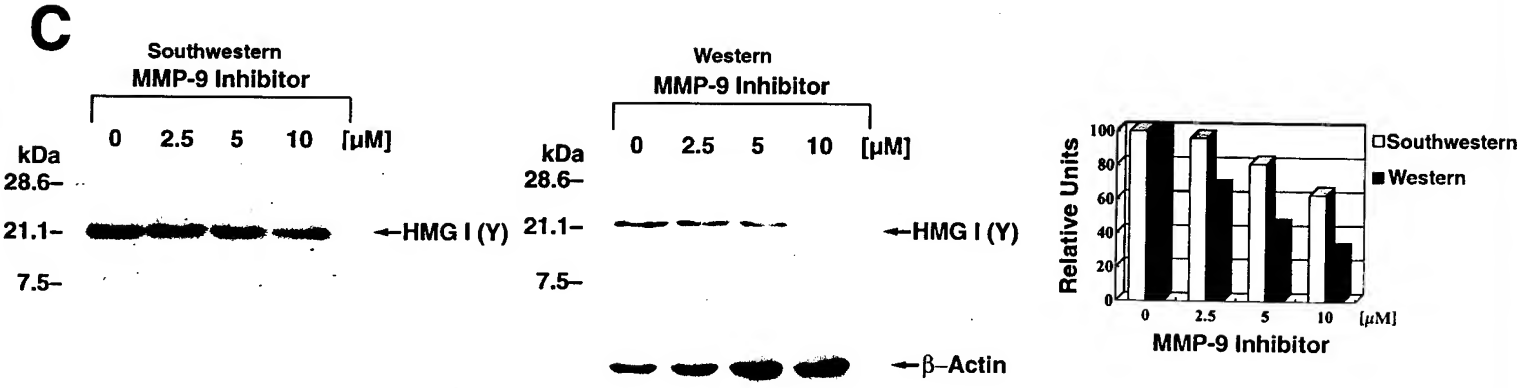



Figure 5